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Cross-species amplification of microsatellite DNA in Old World microtine rodents with PCR primers for the gray-sided vole, *Clethrionomys rufocanus*

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Abstract. Applicability of seven primer sets, originally designed for polymerase-chain-reaction (PCR) amplification of microsatellite DNA in the gray-sided vole, *Clethrionomys rufocanus*, was examined in other 12 microtine species from three genera (*Clethrionomys*, *Eothenomys* and *Microtus*). Of the primer sets used, one distinctly amplified PCR products in all the species examined. Three sets gave PCR products in all but one species. The remaining three sets failed to amplify any products in several species. Non-amplification occurred mostly in *Microtus* species, although two primer sets were not available for two *Clethrionomys* species. Since most amplified loci showed allelic variations, the present primers are useful for molecular ecological studies of related microtines, especially *Clethrionomys* and *Eothenomys* species.

Key words. *Clethrionomys*, *Eothenomys*, microsatellites, *Microtus*, PCR primer.

Microsatellite loci, which consist of tandem repeats of short DNA sequence motif (≦5 base-pairs), are highly variable in repeat number, thereby providing an excellent molecular marker for both ecological and population genetic studies (Burke *et al*. 1992, Queller *et al*. 1993). Genotyping at microsatellite loci facilitates assessment of paternity (Morin *et al*. 1994b, Sillero-Zubiri *et al*. 1996) or relatedness (McDonald and Potts 1994, Blouin *et al*. 1996, Ishibashi *et al*. 1997), and also allows to summarize the genetic structure within or among populations (Morin *et al*. 1994a, Paetkau *et al*. 1995, Lade *et al*. 1996).

Microsatellites can be amplified from a minute amount of DNA using the polymerase chain reaction (PCR) technique (Litt and Luty 1989, Tautz 1989, Weber and May 1989). Hair roots (Washio 1992, Morin *et al*. 1994a), bones (Taberlet and Fumagalli 1996) or feces (Tikel *et al*. 1996) can all be used as sources of DNA, if necessary. PCR-based analysis has a great advantage over conventional allozyme analysis, because of the high resolution and because

sample collection is none, or less, invasive.

Microsatelites are thought to be localized in rapidly evolving non-coding regions, and hence cross amplification is generally restricted to closely related species (Schlötterer *et al.* 1991, Coltman *et al.* 1996, Kayser *et al.* 1996, Valsecchi and Amos 1996). In this study, a cross-species microsatellite amplification was conducted in 12 species of Old World microtine rodents. Seven primer sets originally designed for the gray-sided vole, *Clethrionomys rufocanus*, in Hokkaido, Japan, were used. So far microsatellite loci have not been cloned in other microtines, thus cross amplification could justify the applicability of these microsatellite primers in the species examined.

Materials and Methods

Seven microsatellite primer sets, designed for *Clethrionomys rufocanus* in Japan, were used in this study (Table 1). They consisted of primer sets for five loci previously cloned, MSCRBs-1 to -5 (Ishibashi *et al.* 1995), and two further loci, MSCRBs-6 and 7, newly cloned from the *C. rufocanus* genomic library and sequenced as described by Ishibashi *et al.* (1995). One of the paired primers was newly designed for two loci, MSCRBs-2 and -5, so as to shorten the size of PCR product. For MSCRB-3, one of the paired primers was also redesigned so as to avoid non-amplification which is caused by base substitutions near the CA- and GA-repeats (Ishibashi *et al.* 1996). Cross-species amplification was performed using one to six individuals from each of 12 species from the three genera, *Clethrionomys*, *Eothenomys* and *Microtus* (Table 2). Three *Clethrionomys* species captured in three widely different localities, Japan, Finland

Table 1. Microsatellite primer sets used in this study, including those for the previously described loci, MSCRBs-1 to 5 (Ishibashi *et al.* 1995) and newly cloned loci, MSCRBs-6 and -7.

Locus	Repeat structure[a]	Primers (5'-3')	TA[b]	Product size[c]
MSCRB-1	$(AC)_{24}$	AGTGTTTGGAAGCCATGCGGTA CAGGAGCTTCATGGCTGGAATA	58	150–270
MSCRB-2	$(AC)_{23}$ with several short AC-repeats	AAGGGTGAGTATGCCAATCA TCTCAGATTCTGTGATATGCTGTC[d]	48	100–200
MSCRB-3	$(CA)_{19}(GA)_{24}$	CATGACCTTCTATTTCTGTCAG CTCTAGCATGATGTTACTGT[d]	48	250–350
MSCRB-4	$(CA)_{20}$	GTGCTGCTTACTGGCTTCTTGT CCTGAGTTGTATAAGAAAGCAGGC	60	70–130
MSCRB-5	a mixture of CA-, ATAC- and ATGT-repeats	GGTTGGTGTTTGCATTTAGG CGTCTGGGTTTTACATCTGA[d]	54	130–230
MSCRB-6	$(AC)_{12}(AG)_{25}$	TATAATAGATTTGAGTATCTGC GATGTCCATCAAGTTAATCGT	52	150–220
MSCRB-7	$(AC)_{20}$	GTTTTATGTTAGTCTCATCTG AGGCAATCCTGGTGAGTAACA	52	80–150

[a]Nucleotide sequence of the clones obtained from the Japanese *C. rufocanus* genomic DNA library, [b]Annealing temperature in PCR (°C), [c]Estimated PCR product size for all the species examined in this study (in base-pairs), [d]The primer sequence differed from that previously described by Ishibashi *et al.* (1995).

Table 2. Cross-species amplification with seven pairs of *C. rufocanus* microsatellite primers.

Genus	Species[a]	Location	N[b]	Locus[c]						
				MSCRB-1	MSCRB-2	MSCRB-3	MSCRB-4	MSCRB-5	MSCRB-6	MSCRB-7
Clethrionomys	*C. rufocanus*	Japan	>5	H/+	H/+	H/+	H/+	H/+	H/+	H/+
		Finland	5	H/+	H/+	H/+	H/+	H/−[d]	H/−	H/+
		Norway[e]	5	H/+	H/+	H/+	H/+	H/−[d]	H/+	H/+
	C. glareolus	Finland	5	0	H/+	H/+	H/+	L/+	H/+	0
		Norway[e]	5	L/+	H/−	H/+	H/+	L/+	H/+	0
	C. rutilus	Japan[e]	5	0	H/+	H/+	H/+	H/−	H/+	0
		Finland	5	L/−	H/+	H/−	H/+	H/+	H/−	0
		Norway[e]	5	L/−	H/+	H/−	H/+	H/+	H/−	0
	C. rex	Japan	5	H/+	H/+	H/+	H/+	H/+	H/+	H/+
Eothenomys	*E. andersoni*	Japan (Tohoku)	5	H/+	H/+	H/+	H/+	H/+	H/+	H/+
		Japan (Kii Pen.)	3	L/+	H/+	L/+	H/+	H/+	H/+	L/+
	E. smithii	Japan	5	H/+	H/+	H/+	H/+	H/+	H/+	H/+
	E. regulus	Korea	5	H/−	H/+	H/+	L/−	H/+	H/+	H/+
Microtus	*M. montebelli*	Japan	6	0	H/+	0	H/+	H/+	H/+	L/−
	M. arvalis	The Netherlands	1	0	H/+	L/+	0	H/+	0	H/+
	M. kikuchii	Taiwan	1	0	H/+	0	H/+	L/−	H/+	H/−
	M. melanogaster	Taiwan	2	L/−	L/+	L/−	H/+	H/+	H/+	0
	M. nivalis	Swiss	1	H/−	0	0	H/−	H/−	H/−	H/+
	M. oeconomus	Norway[e]	4	0	H/+	0	H/+	H/−	H/+	H/−

[a]Species names follow Abe *et al*. (1994) for Japanese species and Corbet and Hill (1991) for all others, [b]Number of individuals analyzed, [c]Abbreviations; 0: not amplified (no product or smear), H: amplified at higher annealing temperature, TA (see Table 1), L: amplified at lower annealing temperature, TA−10, −: monomorphic, +: polymorphic, [d]Non-amplification in some individuals (see text for detail), [e]Laboratory-bred animals.

and Norway, were also examined for a possible variation in the applicability of these microsatellite primers (Table 2). DNA was isolated from each animal using the conventional phenol/chloroform method (Sambrook *et al*. 1989).

The PCR amplification was carried out in 10 μl of reaction mixture containing 50 mM of KCl, 1.5 mM of $MgCl_2$, 10 mM of Tris-HCl (pH 8.3), 0.2 mM of dNTP, 0.25 μM of each primer, and 0.25 unit of *Taq* DNA polymerase (TaKaRa). About 30 ng of genomic DNA was used for each reaction. After denaturation at 93°C for two minutes, the reaction was carried out for 30 cycles under the following conditions using a DNA Thermal Cycler PJ2000 (Perkin Elmer Cetus) ; 93°C for 30 sec, TA°C (see Table 1) for 20 sec, and 72°C for 20 sec. TA of each primer was optimized to amplify apparent PCR products in Japanese *C. rufocanus* after calculating with the formula : $69.3+0.41\times$(% of GC content)-650/(primer length) (Mazars *et al*. 1991). When amplification failed in species other than Japanese *C. rufocanus*, lower annealing temperature by 10°C, *i.e.*, TA-10, was adopted so as to allow for mismatches in the primer sequence in the subsequent trials.

The PCR products were electrophoresed in a 3% agarose gel and an 8% non-denatured polyacrylamide gel in order to examine the results of amplification and allelic variation. When amplification in a species did not result in any products, or showed only a smearing pattern, under the above PCR conditions, the result was categorized as "not amplified". If all individuals examined showed a single band only, such a species was categorized as "monomorphic". If two bands of similar size and amount were apparent in one or more individuals, then the species was categorized as "polymorphic".

Results and Discussion

Of seven microsatellite primer sets used, one provided apparent PCR products in all twelve species examined (MSCRB-5, Table 2). Three sets (MSCRBs-2, -4 and -6) gave products in all but one species. The remaining sets (MSCRBs-1, 3 and 7) failed to amplify any products in several species (see Table 2). When amplification was performed with the primer set for MSCRB-3 under the lower annealing temperature, ladder-like band patterns were observed from low to high molecular weight regions. Despite the many spurious bands, we categorized them as "amplified" if the ladder included an apparent band(s) of the molecular size similar to other microtines' products. Non-amplification occurred mostly in *Microtus*, although no apparent product was amplified with the MSCRBs-1 and -7 primers in either *C. rutilus* or *C. glareolus*. In all *Eothenomys* species, products were obtained from all seven primer sets under higher or lower annealing temperature (Table 2).

Non-amplification of microsatellite loci may occur as a result of nucleotide sequence variation (*e.g.*, base substitution, deletion and/or addition) within the priming site for PCR amplification. Therefore, the observed non-amplifications could be due to variation within the priming sequences. Furthermore, in the present study, no PCR products were observed in five of ten

Scandinavian *C. rufocanus* at MSCRB-5 (Table 2). Since allelic variation at the locus is very small in Japanese *C. rufocanus* (Ishibashi *et al.* 1995), these five individuals may be homozygous for a non-amplifying (null) allele with sequence variations in the priming site. Although such null alleles were not detected in microtines oher than the Scandinavian *C. rufocanus*, it is clearly important to pay attention to the possible presence of null alleles especially when using heterologous microsatellite primers (Paetkau and Strobeck 1995, Pemberton *et al.* 1995).

Despite the allelic variation in most amplified loci in each species, interpretation must be made with some caution. In the present study, "polymorphic" and "monomorphic" species are arbitrarily defined on the basis of the number of alleles (bands) in the limited number of DNA samples examined (Table 2). For *C. rufocanus*, *C. glareolus*, *C. rutilus* and *M. oeconomus* from Norway, and *C. rutilus* from Japan, the DNA samples used were extracted from laboratory-bred individuals (Table 2). These animals might have lost heterozygosities at some loci by chance during laboratory breeding. The observed monomorphic band patterns at several loci may not, therefore, indicate the real situation in natural populations.

The present study, though preliminary in nature, demonstrates that most PCR primer sets for *C. rufocanus* microsatellites are useful for detecting allelic variations in related microtines, especially in *Clethrionomys* and *Eothenomys* species. Given the small sample size and the non-systematic collection, further examinations are required to clarify the presence of null alleles and of allelic variation in each population or species of interest.

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